From Department of Oncology-Pathology
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# EVALUATION OF POSTMORTEM TOXICOLOGICAL RESULTS

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# Evaluation of postmortem toxicological results

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To Kristina and Edwin*

# ABSTRACT

Postmortem diagnosis of fatal intoxications is complicated. Autopsy findings indicative of an intoxication related death are scarce and mostly unspecific. Instead, the diagnosis is more dependent on the circumstances surrounding death, and on the toxicological results. In order to correctly evaluate the toxicological results, the forensic investigator needs to know the concentrations of various substances that can be considered "normal" and which concentrations that may be "lethal". While a wealth of scientific data exist concerning concentrations and effects in experimental animals and in living human subjects, these data cannot simply be translated into postmortem concentrations. Due to various changes occurring already in the early phase after death, the blood postmortem concentration of a substance postmortem often does not mirror the concentration antemortem. In order to correctly evaluate postmortem toxicological results there is a need for postmortem reference values, allowing for the separation between the lethal and non-lethal concentrations.

Toxicological data based on the analysis of whole blood from the femoral vein collected at autopsies conducted between 1992 and 2010 were used to establish multiple categories of reference concentrations; Group A (intoxications with a single substance only), Group B (mixed intoxications) and Group C (controls). A well-defined automated selection process followed by a standardized multi-reviewer process and a case-by-case evaluation were applied to generate reference values for each group.

Paper I-IV present postmortem reference concentrations of 48 substances. In most cases there are separate reference concentrations for different types of intoxications (group A and group B) as well as controls (group C). In general, there is a substantial overlap in concentrations between the two intoxication groups, whereas the controls often show much lower levels.

In addition to the establishment of postmortem reference concentrations the different studies included in this thesis provide some additional information. Paper I presents a fatal toxicity index of sedative and hypnotic drugs; Paper II presents the occurrence of antipsychotics among different manners of death; and in Paper III an attempt is made to assess differences in concentrations between acute, acute-on-chronic and chronic intoxications. Further, Paper IV presents the impact of sample size on the precision, power and risk for type 1 error of postmortem reference concentrations. Using the method presented in paper I-IV, >5 cases in each group are needed to reduce the risk of type 1 error (<5%) and >10 cases in each group is needed to have a high power (>0.95). It is suggested that a target number of between 20-30 cases in each group provides a reasonable stability of the reference concentration.

# LIST OF SCIENTIFIC PAPERS

I. Anna K Jönsson, **Carl Söderberg**, Ketil A Espnes, Johan Ahlner, Anders Eriksson, Margareta Reis, Henrik Druid.

*Sedative and hypnotic drugs – Fatal and non-fatal reference blood concentrations*

Forensic Sci Int. 2014;236:138-145

II. **Carl Söderberg**, Emma Wernvik, Andreas Tillmar, Robert Kronstrand, Margareta Reis, Anna K Jönsson, Henrik Druid

*Antipsychotics – Postmortem fatal and non-fatal reference concentrations*

Forensic Sci Int. 2016;266:91-101

III. **Carl Söderberg**, Emma Wernvik, Anna K Jönsson, Henrik Druid

*Reference values of Lithium in postmortem femoral blood*

Forensic Sci Int. 2017;277:2017-2014

IV. **Carl Söderberg**, Andreas Tillmar, Anna Johansson, Emma Wernvik, Anna K Jönsson, Henrik Druid

*The importance of sample size with regard to the robustness of postmortem reference values*

Manuscript

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| C/P | Ratio between central blood (C) and peripheral blood (P) concentration |
| L/P | Ratio between liver tissue (L) and peripheral blood (P) concentration |
| LOQ | Limit of quantification |
| Vd | Volume of distribution (kg/L) |
| pKa | Disassociation constant |
| Kp | Partition coefficient |
| r | Correlation coefficient |
| SMR | Standardized mortality ratio |

# 1 BACKGROUND

## 1.1 WHAT IS AN INTOXICATION?

According to Stedman's Medical Dictionary the term intoxication is defined as a synonym to the term poisoning. Poisoning in turn refers to the state of being poisoned. More clarity results from reviewing the term poison in itself, which is defined as "any substance, either taken internally, or applied externally, that is injurious to health or dangerous to life". A fatal intoxication would then be an intake of poison leading to death [1].

While the definition of the term poison might seem narrow since, at a first glance, not all substances seem harmful to health and life. Things are sadly not so simple;

*"All things are poison, and nothing is without poison, the dosage alone makes it so a thing is not a poison."*

The above quote is attributed to Paracelsus (born Theophrastus von Hohenheim), a Swiss scientist during the German renaissance. He is credited as the father of toxicology for his work on the dose-response relationship [2]. To illustrate his point it is worth noting that even water and oxygen, vital to all humans, can result in death if the dose is high enough [3, 4].

If anything given in a sufficiently high dose can be a poison, it is of utmost importance to know the difference between a harmless and lethal concentration.

This thesis deals with postmortem reference values of drugs, a standardized method for producing them, and their strengths and limitations.

## 1.2 GENERAL BACKGROUND

Since ancient times people have always tried to understand death and the reasons by which it occurs. According to Sydney Smith [5], forensic medicine may be defined briefly as consisting essentially of that body of medical and paramedical scientific knowledge which may be used for the purposes of administration of the law.

The first known textbook on the investigations of suspect death, Hsi Yüan Lu (lit. the "washing away of wrongs"), is of Chinese origin and was published in the thirteenth century AD. The text goes into detail regarding a plethora of questions of forensic medicinal importance, such as strangulation, drowning and poisoning [5]

The first known medico-legal expert can be found in ancient Egypt in the form of Imhotep, Grand Vizier to King Zozer, 3000 BC [5]. It is known that ancient Egyptians performed autopsies and priests made determinations regarding if a death was natural or not [6]. Indeed, the epics of Homer lauds the wealth of knowledge regarding poisons gained from the Egyptians [5]. The literal meaning of the word autopsy is to see "for one's self", to "make a personal inspection". In common usage the term means to dissect a dead body to determine the nature of a disease or the cause of death [6].

While poisons and toxins were known in the ancient world, the techniques for detection of drugs in cadaveric samples did not start to develop until a couple of hundred years back. Considered the founder of modern toxicology, Mathieu Orphila, introduced the analytical foundations of the field in his "Treatise in Poisons" published 1814 [5, 7]. However, it took until the first half of the twentieth century before forensic toxicology emerged as its own branch of the forensic sciences [7].

Toxicology is the discipline that strives to understand the harmful effects of substances and exposures on humans, animals and the environment. Forensic toxicology deals with these questions in the legal context, with postmortem toxicology focusing on questions of toxicological importance in death investigations.

### 1.2.1 Intoxications from a clinical perspective

From a clinical standpoint, when dealing with intoxications among the living, the treatment of a patitent's symptoms takes precedence over identifying the specific agent involved. General therapeutic regimens are used (active charcoal, gastric lavage and/or antidotes to broad classes of substances) alongside interventions to stabilize of the patients vital parameters (airway, breathing and circulation) [8]. Recognition of a specific toxidrome (collections of symptoms typical of intoxication with a specific class of substances) will determine the choice of drug treatment and other measures. Specific laboratory testing for the involved substances or class of substances are useful as long as it can highlight the need for more specific treatment [8, 9]. When the intention is to save lives, the patients symptoms and their treatment are most often a higher priority than the identification of the specific substance (and concentration) involved.

### 1.2.2 Fatal intoxications on the autopsy table

Fatal intoxications are often difficult to diagnose based on autopsy findings alone. Known clinical symptoms might provide information with regard to sample collection and analytical strategy, but clinical symptoms can be unspecific or masked by disease. The vast majority of poisonings with pharmaceutical drugs will leave no characteristic findings at autopsy. Indeed, the most common findings are organ congestion and pulmonary edema, which are quite common in all kinds of deaths [10].

There are exceptions though. Residues of powder or colored material might indicate tablet or capsule remains. Cyanide has a smell of bitter almond. Carbon monoxide poisoning causes a cherry red to light red color of the blood and hence also the livores [10]. Noncardiogenic pulmonary edema resulting in heavy, enlarged lungs stiff with edema and congestion along with froth in the airways is typically found in opioid toxicity deaths [11]. Liver necrosis can be the result of paracetamol (acetaminophen) intoxication [12]. The unexpected finding of pulmonary embolism in a young subject may raise suspicion of an effect of antipsychotics, and in the past of combined oral contraceptives, both known to increase the risk of thromboembolism [13, 14], and a massive spontaneous brain haemmorhage, or gastro-intestinal bleeding may indicate intake of anticouagulants [15].

The above are a few examples, and while they might at first seem specific they can rarely be used in isolation to make the correct diagnosis in the vast majority of intoxication cases. While the smell of bitter almond is a specific finding, when present, in the case of cyanide poisoning one must remember that hydrocyanic acid does not smell like bitter almond to many persons [16] and it is possible to have a cyanide poisoning without anyone noting the odor of bitter almond since only 40-60% of the population possesses the gene necessary to detect the smell [17].

In the end, the best way to diagnose intoxications is by correct sampling, toxicological analysis and a reasonable evaluation of the results. However, in order to evaluate the concentration of a substance in a postmortem cause, the investigator needs to know if the concentration is high or low, normal or toxic. To this end, postmortem reference concentration data are critical.

### 1.2.3 Fatal intoxications from an epidemiological perspective

The adverse effects of drugs and drug dependence are a worldwide problem. While detailed global data are hard to come by, some general trends can be observed. According to a recently published article, summarizing 282 causes of death worldwide, unintentional poisonings and self-harm (excluding deaths due to firearms) comprised 0.9 and 9.2 per 100 000 deaths respectively in 2017 [18]. Using a more narrow perspective, a study focusing on illicit drugs (opioids, amphetamines, cocaine and cannabis), reported that that the all cause mortality comprises an estimated 197,000 deaths annually (including 32,000 due to suicide and 69,000 due to opioid overdose mortality) in 2000, a large increase from the 100,000 estimated deaths in 1990 [19].

Regarding substances causing intoxications, the picture is different in different parts of the world. Outside the western world intoxication by pesticides dominates in Asia (except China), and is second to pharmaceutical drugs in parts of Latin America and sub-Saharan Africa [20, 21]. In the USA and Europe non-pesticide (e.g. drugs illicit or not) poisonings outnumber poisonings by pesticides among suicides [21]. According to 2015 data from the European Centre for Drugs and Drug Addiction (EMCDDA) 17.3 deaths per million population are drug induced in the European Union [22], although this figure refers mainly to unintentional poisonings indicating that this number would be higher if suicidal intoxications were included to a larger extent.

In the Nordic countries there have been periodical reviews of deaths among drug addicts [23-26], with the most recent being based on data from 2012 [27]. While the Nordic countries have a slightly different panorama of drugs involved, some general trends emerge. The most common ascribed main intoxicant, based on the most recent data, is opioids (e.g. heroine/morphine, tramadol, codeine) with either benzodiazepines (and related substances) or other centrally acting drugs (e.g. amphetamine, GHB and new psychoactive substances) following close behind. However, other drugs (i.e. non-illicit drugs or non-scheduled drugs) are a very common finding in drug-addict intoxication deaths, comprising 40-73% of all

analytical findings. In addition poly-drug use was a common finding with the median number of drugs per case (excluding alcohol) being between 4 and 5 in each country, spread among both illicit drugs and medicinal drugs [27].

Thus, in all cases of fatal intoxications it is important to know which substance(s) have caused or contributed. In all death investigations it is equally important to be able to rule out intoxication.

## 1.3 POSTMORTEM TOXICOLOGY

In the clinical world, before a drug is released to the market, extensive testing is required. The putative drug will first undergo in vitro and then experimental testing in animal models before moving on to testing in humans. This testing generates both pharmacokinetic and clinical data that can be useful. Over time side effects of various kinds, harmful and not harmful, are discovered and can be taken into account in treatment. Hence during clinical trials you have access to all this information, together with the specifics in the individual case (such as dose given and the status of patient, and during certain phases also the blood drug concentration) [28].

Next, after the drug has been released on the market, a much larger number of patients will receive the drug, and hence more data on side effects will be reported. This information will typically be limited to the character and degree of the particular side effect and the dose, but not the blood drug concentration [28].

In the postmortem setting the situation is often markedly different. Since postmortem toxicology deals with deceased subjects there is often a lack of information; it might not be known how much of a given drug was ingested (and not even which drugs are suspected) and it is not known if the drug produced any side effects. Hospital records might be lacking or unavailable. Circumstances surrounding the death might be unknown or unclear. In the worst case scenario the only information available is a concentration and a various autopsy findings.

The field of postmortem toxicology has the aim to detect and evaluate effects of detected drugs, and thus to provide guidance in a investigation. However, beyond the difference in available information in a clinical case in and in a postmortem case, there are several factors that make postmortem toxicology an especially challenging field.

### 1.3.1 Important pharmacological terms in postmortem toxicology

When evaluating postmortem concentrations it is important to know how a drug arrives at its destination in the body, and to what extent it is present in different tissue compartments as related to the blood concentration.

When a substance is ingested it must move from its site of administration into the central compartment (blood). Along the way it must cross membrane barriers (e.g in the gastrointestinal tract) and may be subject to metabolic degradation (e.g in the liver). Hence,

not all of the ingested substance reaches the blood stream. The fraction of ingested drug that reaches the circulation is defined as the bioavailability (F) as follows:

$$F = \frac{Quantitiy\ of\ drug\ reaching\ systemic\ circulation}{Quantity\ of\ drug\ administered}$$

where $0 < F \leq 1$. For example, in the case of intravenous administration, all of the drug is directly injected into the systemic circulation resulting in F=1. In the case of oral administration the F is much more variable, and can be as low as a few percent and as high as an intravenous injection depending on the properties of the particular substance [28].

A drug is either passively transported across a membrane along a concentration gradient or actively transported by various active membrane transport proteins. The ease by which substances can cross membranes in the body passively is dependent on multiple factors. Many drugs are weak acids or bases and are thus present in both a more lipid soluble non-ionized form and a less lipid soluble ionized form. The state of drugs ionization is dependent on its disassociation constant (pKa) and the pH surrounding the drug as defined by the Henderson-Hasselbach equation:

$$\log\frac{[protonated\ form]}{[unprotonated\ form]} = pKa - PH$$

or stated differently for acids:

$$pH = pKa + \log\frac{ionized\ concentration}{non - ionized\ concentration}$$

and for bases:

$$pH = pKa + \log\frac{non - ionized\ concentration}{ionized\ concentration}$$

For example a strong base with a high pKa (e g >8) would be present in the body circulation in a predominantly ionized form at normal body pH, decreasing its ability to passively diffuse across membranes [28, 29].

Apart from pKa, there are two other major factors that determine the permeability of a drug. The first is the molecular weight, with larger (heavier) molecules being less able to cross membranes. The second is lipophilicity. Lipophilicity is expressed as the partition coefficient of n-octanol and water, termed Kp. In general the more lipophilic a molecule is the greater its ability to cross membranes [29, 30].

With regard to postmortem redistribution (see below) it is also important to know to which extent a drug is present in the body (i.e tissues) as compared to the circulation. The volume of distribution (Vd) is the amount of fluid that would be required to contain all drug in the body at the same concentration as the drug in the circulation:

$$Vd = \frac{Amount\ of\ drug\ in\ the\ body}{Concentration\ of\ drug\ in\ blood\ or\ plasma}$$

A high Vd indicates that most of the drug is present outside of the circulation (either at its site of action or in other compartments) [28].

### 1.3.2 Postmortem redistribution

Prior to the late eighties and early nighties sampling in postmortem cases was collected from the heart or from the large vessels, or anywhere else in the body where it was available. In many early studies the source of the blood is not specified. The assumption was that a drug was evenly diluted in the blood. However, there was a growing body of evidence that things were not as simple as they first seemed.

One early example concerned digoxine, which provoked several questions. A study by Vorphal and Coe [31] showed that not only were postmortem concentrations of digoxine in general higher than antemortem values (however, in 2 out 11 cases in which femoral blood was sampled lower concentrations were found), there were also markedly different concentrations depending on where the postmortem blood was sampled.

In 1990 Prouty and Anderson [32] published a large collection of concentrations from different substances, sampled from various parts of the body and at differing times. The study found both time and site dependent differences in the postmortem concentrations. Similar findings was found in a study by Pounder and Jones published the same year [33], who suggested that it might be due to the movement of drugs along a concentration gradient from solid organs with high concentrations into the blood. Pounder and Jones named the phenomenon “postmortem drug redistribution” and called it “a toxicological nightmare”.

Subsequent reviews of the subject [30, 34], now called “postmortem redistribution” (PMR), confirmed the early hypothesis by Pounder and Jones. In general the extent of postmortem redistribution is thought to be dependent on three key factors; proximity to one or more reservoir organs, putrefactive changes and the pharmacokinetic properties of the drug involved.

A reservoir organ is a hollow organ (such as the gastrointestinal tract) or an organ with a high concentrating power (such as the myocardium, the liver or the lungs). From the tissues of high concentration the drug then moves along a concentration gradient to surrounding tissues through blood vessels or by way of diffusion. As stated above a proximity to one of these organs increases the extent of postmortem redistribution, making blood from the heart, thoracic/pulmonary vessels and the inferior vena cava prone to this phenomenon. In addition cell death and autolysis, secondary to the putrefactive process, moves drugs previously sequestered in the intracellular space (mainly lipophilic bases) to the extracellular space and into blood vessels. Apart from factors in the body, the characteristics of each specific drug will influence its susceptibility to postmortem redistribution. The disassociation constant (pKa) is of importance to determine which drugs are stored in the intracellular space. Drugs

with a high volume of distribution (Vd >3 L/kg), which are thus to a large extent stored in body tissues, are suggested to a higher degree diffuse back into blood vessels postmortem, even if there are notable exceptions (such as paracetamol which has a low Vd but still exhibits redistribution). It might also be assumed that the lipophilicity (Kp) is an important factor, with the hypothesis that a high Kp increases the likelihood of redistribution [30, 34]. However, the extent to which Vd and Kp can explain the postmortem redistribution of a substance is variable. In a review by Ferner [35] the association between the ratio of central and peripheral blood (as an indication of the extent of postmortem redistribution) and Vd and Kp was weak ($r = 0.247$ and $r = 0.035$, respectively), showing that postmortem redistribution may be difficult to explain.

The ratio between central blood (such as blood from the heart) and peripheral blood is often used as a measure of the extent to which a give substance is prone to redistribution. The reasoning being that central blood is closer to reservoir organs (see explanation above), and thus more prone to redistribution than peripheral blood, which is more isolated. With regard to peripheral blood, femoral blood is the sample of choice as it is the least effected by redistribution [30, 34]. A ratio between central and peripheral blood (C/P) of >3.0 is suggested to be indicative of a potential for postmortem redistribution, while a ratio of 1.0 or less indicates the opposite. However, this leaves substances between these ratios in a grey area [30, 36].

In order to provide better estimates, other ways to estimate of postmortem redistribution have been suggested. McIntyre *et al* [36, 37] have suggested that the ratio between drug concentrations in liver tissue and peripheral blood (L/P) is a more accurate measure, where a low ratio (<5) indicates that a substance is not prone to postmortem redistribution, while a high ratio would (>20-30) indicates the opposite. As an example hydroxyzine, which has a C/P ratio close to 1, has a L/P ratio of ≈14 suggesting a moderate propensity for redistribution (based on the L/P ratio but not based on the C/P ratio) which would align more closely with the pharmacokinetic aspects of hydroxyzine (liphophilic and a high Vd) [37].

Another approach is to measure the concentration in a different matrix. The brain has been suggested as an alternative, justified by the fact that the brain is anatomically isolated from reservoir organs, and has lower metabolic activity and less putrefaction than blood [38]. A series of Danish studies [39-42] have explored concentrations and the ratio between femoral blood and brain tissue (grey matter from the frontal cortex) and have provided reference values for a variety of substances. However, it is important to note that some substances are unevenly distributed in the brain [43], introducing a possible source of error unless sampling is standardized.

In summary it can be said that antemortem and postmortem drug concentrations are, in general, not the same. A variety of factors contribute to this problem. In order to minimize this error sampling is recommended that the sample is collected from a peripheral source of blood (femoral blood) and the result being evaluated with care and with regard to circumstances of the case and the specifics of the drug in question.

### 1.3.3 Postmortem Interval (PMI) and stability

In addition to the phenomenon of postmortem drug redistribution, the elapsed time between death and sampling is an additional factor to take into consideration. An illustrative example of this is a study by Saar *et al* [44] which showed that there was a marked difference in the concentration in peripheral blood of a selection of antipsychotics between the admission to the mortuary and the time of autopsy. Indeed, both increases of concentration of over 100% (chlorpromazine and olanzapine) and decreases of up to 43% (9OH-risperidone) were shown. A similar study by Gerostamoulus *et al* [45] also showed time dependent changes of postmortal concentrations for a variety of substances. Zilg *et al* [46] additionally showed that the postmortem redistribution is more pronounced at longer postmortem intervals. Thus postmortem change is not static, but develops over time. This complicates the evaluation of postmortem toxicological results, especially when the postmortem interval is unknown.

Drugs are also not necessarily stable in postmortem blood. Postmortem degradation of drugs may occur because of metabolic factors in the body or because of the chemical (in)stability of the drug in question [47].

Metabolic factors include endogenous enzymes, many of which lose their activity not in the moment of death but during the first days of the postmortem period [48]. For example, in a study regarding postmortem change of drug metabolizing enzymes in rat liver it was found that the enzyme NADPH-cytochrome P-450 reductase, which is of key importance in the metabolism of many drugs in humans, lost about 60% activity during the first 6h postmortem, and over 90% during the first 48 hours [49]. Metabolic factors also include the activity of bacteria. A well-known example is that of the nitrobenzodiazepines (nitrazepam, flunitrazepam and clonazepam) which are very rapidly metabolized into their 7-amino metabolites by the activity of enteric bacteria [50, 51].

Chemical instability, in which a parent substance and/or a metabolite is degraded or reformed, is also a factor that needs to be taken into account. For example, heroin is rapidly metabolized to 6-monoacetylmorphine (6-MAM) and then on to morphine. Morphine is in turn metabolized in the liver into other substances such as morphine-6-glucoronide (M6G) and morphine-3-glucoronide (M3G). In the postmortem setting 6-MAM is unstable in alkaline aqueous solutions and the glucoronide metabolites may be hydrolyzed back to morphine, making evaluations based on the ratios of metabolites difficult. Amphetamine, while in itself stable postmortem, has unstable metabolites which may degrade to form amphetamine. The antidepressant fluoxetine has shown to undergo significant degradation in room temperature [48]. Volatile compounds (aerosol propellants, anesthetic gases, carbon monoxide, ethanol and organic solvents) are unstable when stored at room temperature, with significant degradation after a few days of storage [47].

The impact of both metabolic and chemical factors on the postmortem change of many drugs also depends on the temperature of their storage. In general, the postmortem degradation of a

drug is due to hydrolysis or oxidation/reduction processes and can be slowed down or halted by decreasing their storage temperature [47, 48]. In a stability study of 46 drugs in postmortem blood, most of them remained stable at -20 ºC when combined with an added preservative (potassium fluoride) [52]. In a study of 30 antipsychotic drugs it was found that while the majority was stable, numerous substances were unstable when stored in 4 ºC and 20 ºC with stability increasing at -20 ºC to -60 ºC storage temperatures [53]. As a general recommendation forensic samples should at least be stored in a refrigerator and preferably frozen at -20 ºC [54]. However, normally, until found, recently deceased bodies are often found in room temperature making reference information about drug stability in room temperature particularly important.

### 1.3.4 Circumstances of the deceased, of the cause of death and resuscitation

Hospital care, resuscitation and the cause of death in itself can impact the interpretation of postmortem drug concentrations.

In a review by Richardson of the myriad of issues affecting the interpretation of postmortem toxicological results, aptly named “Pitfalls in forensic toxicology” [55], the impact of hospital care with the treatment with intravenous fluids is discussed. Naturally adding large amounts of fluids to the body can dilute, or render tissues devoid of, detectable drug concentrations. The pharmacokinetics of critically ill patients with impaired cardiac output, blood pressure and ventilation in addition to possible acidosis and the effects of disease, are probably different from those seen in study populations seen in most pharmacokinetic studies. Renal disease might affect the excretion of a drug and liver disease might affect its metabolism. [47]. Resuscitation attempts can also impact postmortem redistribution moving central blood more peripherally, and therefore influence the measured concentration of a drug in a peripheral blood sample [30, 34].

Traumatic deaths with extended blood loss might effect the concentration of drugs, as the body tries to adapt to exsanguination, such as the transfer of extravascular fluid into the circulation [56, 57].

Tolerance, implying various adaptations in the response to a drug upon repeated exposures, represent a well-recognized problem in the clinical setting, particularly in the treatment of chronic pain. Tolerance can be said to have developed when it is necessary to increase a dose to obtain the same effect previously obtained with a lower dose. Development (or loss) of tolerance is also a factor that has to be considered in the interpretation of postmortem blood concentrations. One example are opioids, which are known to induce substantial tolerance when used continuously [55]. It has been shown that blood concentrations alone, are not reliable when evaluating possible opioid intoxications [58, 59]. In these cases recent previous exposure must be confirmed or excluded in order to evaluate the impact of an opioid substance detected. One solution when screening for previous exposure in the

timeframe relevant in forensic cases, is hair collected from the vertex or back of the head. Circulating substances in the blood are continuously being absorbed by the growing hair follicle and incorporated into the bulb of the hair root. A certain proportion of the drug incorporated into the growing hair is absorbed from sweat glands surrounding the follicle. Segmental hair analysis can thus provide a temporal map of recent substance intake (or lack thereof). A lack of exposure (suggesting abstinence) in the innermost hair segment can then be used together with the presence of the drug in blood to support a suspicion of intoxication as the cause of death [59, 60]

The disadvantage of using hair to indicate the presence or absence of tolerance by way of previous exposure is that tolerance may change in a shorter period of time than that covered by segmental hair analysis. One way of approaching this problem would be to use pharmacokinetic aspects. In a recent study Selden *et al* have used the different peak times of buprenorphine and its metabolite norbuprenorphine in both blood and urine as an indicator for recent intake of drug prior to death [61].

## 1.4 REFERENCE CONCENTRATIONS

### 1.4.1 General considerations

Due to changes in blood drug concentrations that can occur early after death, reference information about drug concentrations in living subjects is not a reliable source for comparison when evaluating postmortem toxicological results. The forensic community has responded to this problem by building reference values from postmortem material. There are multiple methods of producing reference concentrations, each with strengths and weaknesses. The following section will provide some examples of different types of reference values.

### 1.4.2 Case reports and case series

The most common approach is a descriptive publication of a case or a small series of cases. The advantage of this approach is that there is often ample circumstantial information available, in the form of medical history and autopsy findings, such as a report of a citalopram overdose [62] which includes previous medical history, information from the intensive care unit in addition to the toxicological findings.

One issue with case reports and small series is that in order to gather a body of knowledge you need numerous reports, since each report only contains a single or a small group of cases. When compiling these findings there can be differences in sample site, matrix used (for example plasma, serum or whole blood) and circumstances of the case, which can make generalization difficult. Olanzapine can serve as an example of this issue; one study reported a concentration in heart blood in a cardiac death [63], another reported an intoxication case in heart blood [64] and a third reported an olanzapine related fatality with a blood concentration from an unknown sample collection site [65]. While this is only a small sample with a few

examples, the sources of potential error and variation can add up over time when building a body of reference material based on case reports.

### 1.4.3 Descriptive compilations

One option to increase the number of cases is to extract whole populations from a forensic archive (such as a database), for example extracting all cases in which a specific substance has been found. The advantage of this approach that it, depending on the size of the archive, is easy to present a large number of cases for a variety of substances present in the material. Examples of this approach are the studies by Jones and Holmgren [66] and Launiainen and Ojanpärä [67], respectively, in which they have used national forensic databases from Sweden and Finland. In both studies the upper percentiles of detections in the population ($90^{th}$, $95^{th}$ and $97.5^{th}$) have been presented along with the mean and the median values. This type of reference is excellent if you want to know if the concentration of a substance is high or not with regard to the general body of detections, but does not tell you wherein that range of detections you can find intoxication cases (or normal cases for that matter).

A similar approach is to extract cases from a large archive, as above, but to also divide them according to cause of death. Jones *et al.* [68] used this approach in a recent study where they presented concentrations in deaths attributed to intoxication and subdivided them into single- or multi-drug intoxications and presented mean, median and percentiles as above. This narrows down the presented population and allows for more precision. However, with regard to multi-drug intoxications it is not always clear if the presented drug in question was a key substance (that is actively contributing to the cause of death) or an incidental finding.

### 1.4.4 Evaluated compilations

One way to increase the accuracy of postmortem reference values is to combine the number of cases retrieved from a database with an evaluation of each case. The result aims to be a "best of both worlds" scenario, combining the review of a case report with the numbers from an archive compilation. One example is a review of alprazolam related deaths in Palm Beach County [69], which examines all cases in which alprazolam was found between 2001 and 2003. The study presents concentrations not only in alprazolam intoxication deaths but also in death from natural causes, providing a broad reference for evaluation.

One key advantage of the above study is the reporting of the results in natural death cases; the importance of knowing what is normal. As mentioned previously it is equally important to know which substances have not contributed to an intoxication death as the reverse.

Apart from the studies included in this thesis (see Paper I-IV), and previously published work from the research group of professor Henrik Druid [70, 71], numerous studies from Denmark have used a similar approach [39-42, 72-74]. Both of these groups use a method of evaluating database findings to not only present concentrations in intoxication deaths, but also in deaths with causes of death other than intoxication, which is if great use to the forensic pathologist.

### 1.4.5 The importance of sample size

In the scientific literature on postmortem toxicology there is a paucity of information regarding the impact of the size of your reference sample on the robustness of the presented reference value.

A review article by Drummer [75] touches the subject and acknowledges its importance, but provides no clear data or guidelines. The review of "pitfalls" in forensic toxicology by Richardson [55] mention "insufficient experimental or case data" as one of many factors impacting the reliability of postmortem reference concentrations, but does not provide additional guidance. Other reviews do not mention this aspect at all [10].

However, common sense suggests that a reference concentration based on many observations is more reliable than one based on a few cases. To which extent that reliability varies has so far not been clarified.

## 1.5 SEDATIVES AND HYPNOTICS

Regarding sedatives and hypnotics, including anxiolytics (ATC-code N05B and N05C), there are a variety of drugs of forensic interest, such as benzodiazepines (e.g. diazepam, flunitrazepam and alprazolam), drugs with benzodiazepine-like effect (e.g. zaleplon, zolpidem and zopiklon) as well as non-benzodiazepine hypnotics (e.g. propiomazine).

Benzodiazepines have central nervous system depressant properties. They bind to the $GABA_A$ receptor, which is present on most neurons in the brain and spinal cord. The GABA receptor is heterogenic, i.e. it is built up by five different subunits in a variety of configurations that together form the receptor [76]. The benzodiazepines bind to a binding site between two different subunits of the $GABA_A$ receptor [77]. Upon bindning, benzodiazepines modifies the receptor to increase its response to GABA, resulting in an increased effect for a given concentration of the neurotransmitter. GABA acts as a CNS depressor by hyperpolarizing neurons, increasing their threshold for activation. In a clinical setting increased GABA effects protect against seizures, but also causes sedation, promote sleep and relieve anxiety [78]. The non-benzodiazepine drugs mentioned above with a similar effect (zaleplon, zolipdem and zopiclon) also bind to, and interact with, the subunits of the $GABA_A$ receptor, although at a site different from benzodiazepines proper [79].

Benzodiazepines are one of the most frequently prescribed psychotropic drugs in the world. They are of forensic interest because of their abuse potential, especially in combination with alcohol and opioids. While there are some minor pharmacokinetic interactions between the benzodiazepines and opioids (benzodiazepines being a weak competitive inhibitor of CYP3A4, which metabolizes some opioid drugs), there are mainly pharmacodynamic reasons for combined use. The abusers typically experience an increased subjective effect of a "high" when taking opioids together with benzodiazepines. In addition, opioids and benzodiazepines

together produces an increased effect on respiratory depression than either drug acting alone, which can be fatal [80].

According to epidemiological data from the United States benzodiazepines were involved in 31% of fatal overdoses in 2013. Indeed, during the years 1999 to 2013 the percentage of adults filling a benzodiazepine prescription increased from 4.1% to 5.6%; during the same period the rate of benzodiazepine related deaths increased from 0.58 to 3.07 per 100 000 individuals [81]. According to an Nordic epidemiological study on deaths due to fatal intoxications among drug abusers from 1997 to 2012, benzodiazepines is an increasingly common finding (71-88% of cases as of 2012, with only Iceland breaking the mold at 27%) as well as in the number of cases in which it has directly contributed to the death (16.5% of Swedish cases 2012) [27].

## 1.6 ANTIPSYCHOTICS

Antipsychotics (ATC-code N05A) include a variety of drugs to treat schizophrenia. Antipsychotics can be roughly divided into two distinct groups of drugs; first generation antipsychotics (FGA) and second-generation antipsychotics (SGA). FGAs include, among others, phenothiazines (e.g. chlorpromazine and levomepromazine), butyrophenones (e.g. haloperidol) and thioxanthenes (e.g. flupentixol). SGAs include, among others, clozapine, olanzapine and risperidone [82].

All FGAs have a high affinity for the dopamine $D_2$ receptor with studies showing that they bind tightly and disassociate slowly [82]. Antipsychotics exert an antagonistic effect on the $D_2$ receptor, reducing its signaling. This is important since the postulated hypothesis for the pathological mechanism in schizophrenia is increased postsynaptic $D_2$ receptor sensitivity [83]. However, a high $D_2$ receptor blockade can lead to cognitive impairment and extrapyramidal symptoms (EPS; e.g. dystonia, parkinsonism and tardive dyskinesia). SGAs, also known as atypical antipsychotics, have a broader panorama of receptor affinities binding not only to $D_2$ receptors but also the serotonin receptors (e.g. 5-$HT_{2A}$). It has been suggested that a higher ratio of binding to the serotonin receptor relative to the dopamine receptor explains the increased efficiency and reduced risk of extrapyramidal symptoms of SGAs. However, SGAs carries an increased risk of other adverse effects such as weight gain, diabetes mellitus and prolonged QTc-interval [82].

Globally schizophrenia accounts for 7.4 % of disability-adjusted life years (DALYs, a measurement combining premature mortality and disability) caused by mental and substance abuse disorders [84]. Patients diagnosed with schizophrenia, the main recipients of antipsychotic medication, are also a group of forensic interest. A review showed that patients suffering from functional disorders, particularly schizophrenia and major depression, has an increased risk of unnatural death [85]. Similarly, a Swedish study of patients suffering from schizophrenia between 1973 and 1995 showed a marked increase in mortality for both males and females compared to the general population. Interestingly, from a forensic standpoint, was that the standardized mortality ratio was increased for both natural and unnatural death

(2.8 for males and 2.4 for females), with the highest being suicide (15.7 for males and 19.7 for females) and unspecified violence (11.7 for males and 9.9 for females) [86]. Regarding suicides an international study showed that among suicides in patients suffering from schizophrenia, poisoning accounted for 18.6% of the cases, with hanging and jumping being more common (28.2% and 25.6% respectively) [87].

## 1.7 LITHIUM

Lithium (ATC-code N05AN01) is a type of antipsychotic medication used in the treatment of bipolar disorder.

Lithium is a true mood stabilizer in the sense that is has both anti-manic antidepressant effects, even if the anti-manic effect is more pronounced. However, the specific mechanism behind the clinical effect of lithium is not fully known. At the neuronal level lithium inhibits excitatory neurotransmission (dopamine and glutamate) and increases inhibitory neurotransmission (GABA). However, the picture is more complex since lithium also affects intracellular secondary messenger systems, such as adenylyl cyclase and protein kinase C that may reduce excitatory neurotransmission. In addition it is has been shown that lithium has more general neuroprotective effects in that it reduces oxidative stress, which occurs with multiple episodes of depression and mania [88]. While lithium is an effective mood stabilizer, not all patients show a response to the treatment and the individual response is variable. It has been suggested that the response to lithium has a genetic component, since, for example, good responders more often have family history of bipolar disorder and a twin study showed better effect of lithium if the co-twin also has bipolar disorder. Recently a study showed a locus on chromosome 21 might be involved in the lithium response [89].

Of special importance is that evaluation of lithium concentrations in suspected intoxication cases is dependent on the mode of intoxication. Studies have proposed three different scenarios of lithium intoxication; acute (large intake in the absence of previous lithium treatment), acute-on-chronic (large acute intake in the presence of chronic lithium treatment) and chronic (progressive lithium toxicity following a change in treatment dose, a decrease in renal clearance or due to the presence of certain other medical conditions). Lithium seems to have a reverse tolerance relationship, with previous exposure making an individual more sensitive to lithium toxicity. It is believed that this phenomenon is related to differing baseline levels of lithium saturation in tissues, between the different forms of lithium intoxication [90, 91].

Globally bipolar disorder accounts for 7.0% of disability-adjusted life years (DALYs, a measurement combining premature mortality and disability) caused by mental and substance abuse disorders [84]. As with schizophrenia, patients diagnosed with bipolar disorder have an increased standardized mortality ratio both with respect to natural, but also unnatural, death [92]. International studies show that these patients comprise 2.3–9.6% of all suicidal deaths [87, 93-95]. The standardized mortality ratio for suicide in this population is increased (SMR 22), even when compared with other mental disorders such as major depression

(SMR ≈20). In addition, the ratio of suicide attempts to completed suicides is only 5, compared to 10-20 in the general population [96]. With regard to the suicide method used studies show variable results, indicating that fatal intoxications comprise between 17% and 53% of all suicides [97].

# 2 AIMS

The overall aims of the study were

- to establish postmortem reference values for selected substances
- to use the information in our database to answer additional questions of forensic interest concerning the studied substances
- to investigate the number of cases needed to produce stable and reliable reference values

# 3 MATERIAL AND METHODS

## 3.1 STUDY POPULATION (PAPER I-IV)

In Sweden a physician must certify all deaths. Depending on the circumstances, this doctor should also make a decision as to if the death should be reported to the police. All cases where unnatural death (i.e. when some external factor might be involved) cannot be ruled out are to be reported to the police. In addition, deaths in which the identity is unclear, in cases of suspected medical malpractice and cases of suspected sudden infant death syndrome should also be reported to the police. The police in turn will request a forensic autopsy to be performed by a Forensic Medicine Department of the Swedish National Board of Forensic Medicine.

The Division of Forensic Medicine of the National Board of Forensic Medicine comprises six different Departments of Forensic Medicine from Umeå in the north to Lund in the south. Together they perform between 5000 and 5500 autopsies each year [98]. In each case samples (femoral vein blood is the sample of choice for drug analysis, when available) are taken for toxicological analysis at the national forensic laboratory at the Department of Forensic Toxicology and Genetics. The toxicological results for each case together with the forensic pathological findings are stored in a central database. At the time of writing the database contains about 130,000 autopsy cases, and for most of them there are also toxicological results [99]. The included studies in this thesis are all based on data extracted from this database, although during somewhat different time periods for different substances.

### 3.1.1 Paper I

The population in paper I is comprised of all cases from the years 1992-2006 in which sedative and hypnotic drugs (ATC codes N05B and N05C) and clonazepam (ATC code N03AE01) were found in femoral blood.

### 3.1.2 Paper II and Paper III

The populations in paper II and paper III is comprised of all cases from the years 1992-2010 in which antipsychotics (ATC code N05A), lithium (ATC code N05AN01) and/or alimemazine (ATC code R06AD01) were found in femoral blood.

### 3.1.3 Paper IV

Paper IV comprises all cases from the years 1992-2010 in which one or more of 13 substances (ATC code) were found in femoral blood; alprazolam (N05BA12), amitriptyline (N06AA09), carbamazepine (N03AF01), citalopram (N06AB04), nitrazepam (N05CD02), olanzapine (N05AH03), oxazepam (N05BA04), oxycodone (N02AA05), phenytoin (N03AB02), quetiapine (N05AH04), tramadol (N02AX02), verapamil (C08DA01) and zolpidem (N05CF02).

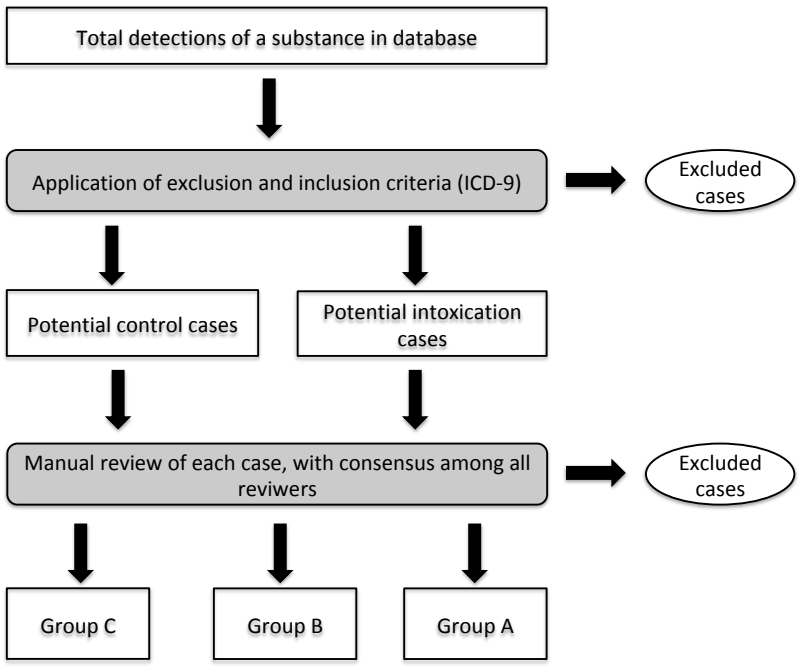
## 3.2 SELECTION OF CONTROL CASES, GROUP C (PAPER I-IV)

The control group (group C) is made up of cases in which intoxication has not played a role in the death. However, not being an intoxication is not the only criterion that has to be met in order for a case to be classified as a C-case. As the representation of what constitutes a "normal" concentration is important for the selection of intoxication cases (see below), it is of utmost importance that the control group is equally reliable. To this end all cases of death in the control group also have to reflect that the deceased had an ability to act (i.e. was not incapacitated by drugs) at the time of death. Typical examples are hangings and suicidal gunshot to the head, which both require active action by the person, hence indicating an ability to perform complex tasks immediately prior to death. On the opposite side of the spectrum there is death by submersion (drowning) that could be related to at least partial incapacitation (e.g. drowning in a bathtub when it cannot be ascertained that the death was a result of drugs and the drowning was secondary, or the opposite).

The cause and manner of death may influence blood drug concentrations. Damage to a reservoir organ, and in particular contact between the gastrointestinal tract and the blood may cause transferal of high drug concentration to the blood and hence falsely high concentration in the blood sampled. Slow exsanguination [56, 57] or hospital care [55] could also impact drug concentrations. Hence, such cases are excluded from the C-group. While detailed information of this kind is not always available in the forensic database directly, each case has to be manually reviewed to screen for these potential sources of error.

Selecting the cases for the control group is thus a multi-step process; Figure 1 provides a schematic overview of the chain of events leading to a confirmed control group case.

**Figure 1.** Schematic workflow of the process used to produce postmortem reference concentrations. Modified from Paper IV



A set of inclusion and exclusion criteria is applied to the broader study population. The criteria has been similar in all included papers, however in paper II and paper III, pulmonary embolism was added as an exclusion criterion for the C-cases because of the increased risk of pulmonary embolism as a side effect of antipsychotic medication [100]. Tables 1 and 2 detail the inclusion and exclusion criteria, based on ICD-9 codes with supplements defined by the Swedish medico-legal society. The resulting population is made of potential group C cases.

**Table 1:** Inclusion criteria for group C based on ICD-9 codes (with supplementary suffices[a])

| Manner of death | Cause of death | Definition |
|---|---|---|
| | 800-959 | Trauma and extraneous bodies in upper and lower airway |
| | 410K[b] | Acute myocardial infarction with rupture |
| | 441A[b] | Dissecting aortic aneurysm |
| | 441B[b], 441D[b] | Ruptured abdominal aortic aneurysm |
| | 994B[b,c] | Drowning |
| E953 | | Suicide, hanging, asphyxiation or suffocation |
| E955 | | Suicide, firearms or explosive substances |
| E956 | | Suicide, sharp force injuries |
| E958 | | Suicide, without further specification |

a Certain suffices are supplements to the ICD-9 codes defined by the Swedish Medico-legal Society and used by the Swedish forensic pathologists to provide more detailed and specific diagnoses
b Included as inclusion criteria if the number of C-cases were less than 200
c only drowning by witnessed jumps into water were included.

**Table 2:** Exclusion criteria for group C based on ICD-9 codes (with supplementary suffices[a])

| Manner of death | Cause of death | Definition |
|---|---|---|
| | 415B[b] | Pulmonary embolism |
| | 852L | Traumatic subdural hematoma |
| | 861K | Bullet wound in thoracic region |
| | 861M | Injuries in thoracic internal organs |
| | 864 | Liver injury |
| | 869 | Multiple internal injuries |
| | 933 | Extraneous body in pharynx and/or larynx |
| | 934 | Extraneous body in bronchi and/or lung |
| | 940-949 | Burns |
| | 991G | Hypothermia |
| | 994B[c] | Drowning |
| E850-E858 | | Accident, intoxications by drugs |
| E880-E888 | | Accident, fall |
| E910 | | Accident, drowning |
| E954[b] | | Suicide, drowning |
| E958A | | Suicide, collision with train |
| E958B | | Suicide, burning |
| E958D | | Suicide, frozen to death |
| E960-E969 | | Murder |
| E980-E989 | | Unclear manner of death |

a Certain suffices are supplements to the ICD-9 codes defined by the Swedish Medico-legal Society and used by the Swedish forensic pathologists to provide more detailed and specific diagnoses
b In paper II-IV Pulmonary embolism was an exclusion criteria for antipsychotics due to the increased risk for pulmonary embolism as a side-effect of treatment [14].
c Death by drowning (994B) was excluded, if unwitnessed (most often deaths in bath tubs).

Multiple investigators then review the potential group C cases independently, mainly to screen for circumstances not immediately apparent from the cause(s) of death in the database. For example; the length of any hospital care is checked, and if the stay exceeded several hours, the case is excluded since the drug levels then may have dropped or been affected by various treatments; cases of drowning and falls are reviewed to remove cases where incapacitation cannot be ruled out; trauma cases are reviewed to remove cases with damage to reservoir organs and risk of contamination. In addition, cases with unexpectedly high concentrations are reviewed to screen for possible misdiagnosed intoxications.

After independent reviews, the investigators meet and discuss cases where classification differ. A case is not included in group C unless all investigators are in agreement that it constitutes a control case. Note that each case can be selected into group C for multiple substances, if there are detections of several of the substances that are reviewed.

## 3.3 SELECTION OF INTOXICATION CASES, GROUP A AND GROUP B (PAPER I-IV)

Intoxication cases are selected from the study population based on the cause of death diagnosis made by the responsible forensic pathologist. As the name suggests the intoxication cases are those cases in which death has been primarily attributed to intoxication by drugs and/or by alcohol. The cases are extracted from the study population based on a set of inclusion criteria based on based on ICD-9 codes with supplements defined by the Swedish medico-legal society. The resulting population is made up of potential group A or group B cases.

As with the control cases, selection of the intoxication cases is a multi-step process, Figure 1 provide a schematic overview of the chain of events leading up to a confirmed group A or group B case. After the automatic selection based on ICD-9 codes (see above) each case is subject to an independent review by multiple observers. In this review the key substance(s) considered responsible for each intoxication case are identified, and the concentration range of group C is here used as a guideline to interpretation. Intoxications in which only a single key substance has been identified are classified as A-cases. Intoxications with multiple substances and/or with an ethanol concentration of >0.1g/dL are classified as B-cases. Cases in which opioid intake might have contributed to the cause of death are excluded as concentrations of opioids are difficult to evaluate because of tolerance. As with the control group, cases that have been subjected to extended hospital care are also excluded.

Similarly to the procedure for the C-cases, the investigators meet and discuss cases, where discrepant classification has been made. A case is not included in group A or group B unless all investigators are in agreement. Note that groups A and B are mutually exclusive, and a case can only belong to one of the groups. However, a single case might contribute to group B multiple times for different key substances.

## 3.4 SELECTION OF LIVING CONTROLS (PAPER I AND II)

In Paper I and Paper II, living controls were included in addition to the postmortem controls described above. We used data gathered from therapeutic drug monitoring and driving under the influence cases to gather a suitable control population.

### 3.4.1 Therapeutic drug monitoring (TDM) cases (Paper I and II)

Concentration data was gathered from therapeutic drug monitoring cases at the Department of Clinical Pharmacology in Trondheim, Norway. A single sample per patient was used. Cases of intentional and unintentional intoxications were excluded, based on the requesting physician´s notes. The TDM data was originally presented as mmol/L and were transformed to the same unit as the postmortem data (µg/g), approximating that 1ml of serum weighs 1 g.

Paper I included TDM data collected between 1999 and 2007.

Paper II included TDM data from variable time periods for different substances; 1999–2006 (chlorpromazine, methotrimeprazine, perphenazine, risperidone, sertindole and zuclopenthixol), 1999–2007 (alimemazine), 1999–2008 (clozapine), 2000–2005 (quetiapine), 2000–2006 (flupentixol), 2000–2014 (amisulpride), 2001–2006 (haloperidol and ziprasidone), 2005–2006 (aripiprazole) and 2005–2007 (olanzapine).

### 3.4.2 Driving under the influence (DUI) cases (Paper I and II)

Concentration data in cases of driving under the influence analyzed at the department of forensic toxicology and genetics in Linköping, Sweden, were compiled. Both studies included cases in which relevant substances had been found. Only one sample per person was included, thus excluding serial offenders.

Paper I included DUI case data collected between 1992-2006.

Paper II included DUI case data collected between 1992-2010.

## 3.5 ANALYTICAL CONSIDERATIONS (PAPER I-IV)

All postmortem samples, as well as the DUI samples mentioned above, were analyzed at the Department of forensic toxicology and genetics in Linköping, Sweden. All substances except lithium were identified and quantified in the same laboratory. However during the time period during which data has been included the methods used and the limit of quantification has varied. Details regarding used methods are specified in each paper. In addition, for each paper in which method and/or LOQ has been changed during the study period, these variations are presented in separate tables.

For purposes of keeping the concentration data comparable over time, the highest LOQ for each substance during the study period has been used as a cut-off. The same "cut-off" LOQ was also applied to DUI and TDM data as mentioned above.

### 3.5.1 External analysis of Lithium (Paper III)

Lithium was analyzed externally during the study period (1992-2010) at Department of Clinical Chemistry at Linköping University Hospital (Sweden), at Jönköping Regional Hospital (Sweden) as well as the Oslo University Hospital (Norway).

## 3.6 FATAL TOXICITY INDEX (PAPER I)

The fatal toxicity index presented in paper I compares the number of intoxications (i.e. group A and B combined) in which a given substance was considered to be contributing to the intoxication death with the sales of the substance during a specific time period.

Sales statistics were defined as the number of defined daily doses (DDD) sold in Sweden during the specific time period. A defined daily dose (DDD) is defined as the assumed mean dose given to an adult on maintenance therapy for the drugs primary indication. The DDD

data was retrieved from Apotekens Service AB, which, during the study period, administered the Swedish prescription database.

DDD sale statistics were available for the whole study period (1992-2006) for all substances except buspirone, clomethiazole, clonazepam, hydroxyzine, propiomazine, zaleplon, zolpidem and zopiclone, for which data only were available during 2000-2006.

## 3.7 MANNER OF DEATH (PAPER II)

The manner of death statistics presented in paper II combined the number of cases in which the substance was detected, the number of intoxication deaths the substance was detected in and the number of intoxication deaths in which the substance was considered as contributing stratified on manner of death.

The external codes (E-series) of ICD-9 were used to define the manner of death as follows:

Suicides: E950-E959, including suicidal intoxication (E950); accidents: E800-E949, including accidental intoxication (E859, E860 and E866); undetermined cases: E980-E989, including undetermined intoxication (E980); and homicides: E969-E969, including homicidal intoxications (E962).

These selection criteria were then applied to the study population as a whole and to the selected group A and B cases, in order to differentiate the intoxications in which a given substance was an incidental finding in which cases it was considered as contributing to the cause of death.

## 3.8 MODE OF INTOXICATION (PAPER III)

Based on the classification by Jaeger *et al* [90] all included lithium cases in group A and group B was classified into acute, acute-on-chronic or chronic intake based on available case information.

## 3.9 VARIANCE OF MEDIAN, RISK OF TYPE I ERROR AND POWER (PAPER IV)

Due to the non-normal distributions of the observed concentrations only non-parametric metrics were applied for the various statistical analyses. For each substance, the impact of the size of the data set was studied by randomly drawing (with replacement) N samples from the original set of concentrations (groups A, B and C). N varied from 1 up to 50. For each sample size (N), the sampling process was repeated 1,000 times.

The type 1 error (different distributions of concentrations) was estimated by statistically comparing the data from the small data set of concentrations with the data from the complete set of concentrations using a Mann-Whitney U-test. A p-value below 0.05 was considered to be a statistically significant difference and the proportion of such differences was estimated for each sample size, N, based on the 1,000 replicates.

If a significant difference was observed between the different groups in the original data set of concentrations, the power to detect that difference with smaller samples sizes was studied in a similar fashion as the type 1 error above. A Mann-Whitney U-test was applied to statistically compare the concentrations for the different groups.

All statistical analyses were performed using the R software v. 3.5.0 [101]

## 3.10 ETHICAL CONSIDERATIONS

The Regional Ethics Review Board in Linköping, Sweden, has approved all included papers (No 2012/343-31).

# 4 RESULTS

## 4.1 POSTMORTEM REFERENCE VALUES (PAPER I-IV)

Paper I-III presents postmortem reference values for 41 substances subdivided into two intoxication groups (group A and group B) and one control group (group C). Paper IV adds additional 7 substances and provides updates from paper I and II to 6 more. In general there is an overlap between the two intoxication groups, but the intoxication groups are in general significantly different than the control group. While tables with the specific reference values for each substance are presented in the different papers, a few examples are shown below (Table 3);

**Table 3**: Selected reference concentrations (μg/g) adapted from Papers II and IV

| **Substance** (Total detections in database) | **Group** | **N** | **10th percentile** | **Median** | **90th percentile** | **P-value** |
|---|---|---|---|---|---|---|
| Citalopram (4384) | A | 70 | 1.09 | 4.45 | 21.23 | vs B <0.001 |
| | B | 377 | 0.70 | 1.40 | 9.04 | vs C <0.001 |
| | C | 916 | 0.10 | 0.30 | 0.70 | vs A <0.001 |
| | | | | | | |
| Thioridazine (412) | A | 19 | 1.34 | 2.40 | 4.28 | vs B 0.16 |
| | B | 40 | 1.09 | 1.80 | 4.05 | vs C <0.01 |
| | C | 41 | 0.20 | 0.40 | 0.7 | vs A <0.01 |
| | | | | | | |
| Ziprasidone (3) | A | - | - | - | - | - |
| | B | 1 | - | 5.70 | - | vs C 1.0 |
| | C | 1 | - | 0.008 | - | - |

## 4.2 MANNER OF DEATH AND FATAL TOXICITY INDEX (PAPER I-II)

Paper I presented a fatal toxicity index of sedatives and hypnotics. Flunitrazepam was the most common key substance in single intoxication deaths (group A) related to sales. If both single intoxications (group A) and multi-substance intoxications (group B) are taken into account hydroxyzine was the most common key substance related to sales.

Paper II presented the occurrence of antipsychotics according to the manner of death. Alimemazine and methotrimeprazine were the most commonly detected antipsychotics across all manners of death. However, the most common key substance was clozapine with a high proportion of detections in suicidal, uncertain and accidental cases.

## 4.3 CONNECTION BETWEEN MODE OF INTOXICATION AND LETHAL CONCENTRATION (PAPER III)

Paper III attempted to investigate whether or not the mode of intoxication affect which concentrations should be considered lethal or not. However, due to the paucity of positive lithium detections no conclusions could be drawn. Figure 2 illustrates the relationship between detections, included intoxication cases and mode of intoxication.

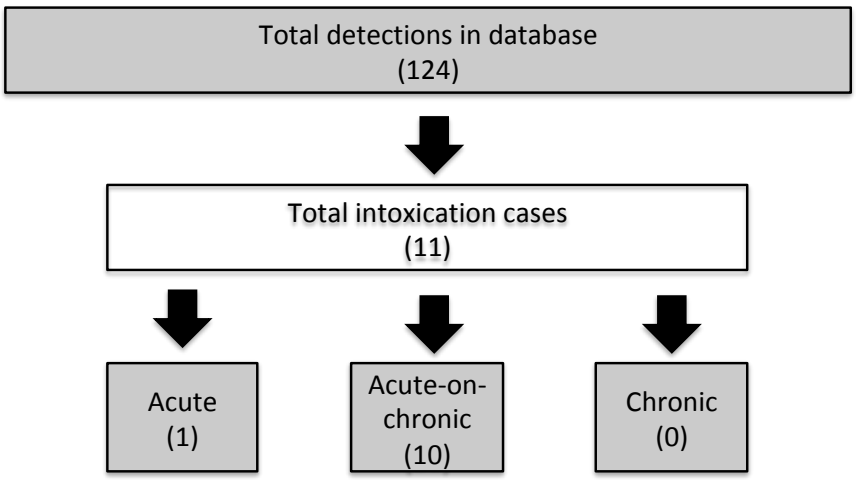



**Figure 2.** Relationship between total detections, included intoxication cases and mode of intoxication. Adpated from paper III

## 4.4 SAMPLE SIZE, POWER AND PRECISSION (PAPER IV)

Paper IV presents data on the impact of sample size on precision, power and the risk of type I errors. In general, > 5 cases per group (A/B/C) are needed to reduce the risk of type I error below 5% and >10 cases are needed to reach a power of >0.95 when differentiating between intoxication (group A and group B) and controls (group C). The general trend is that the precision improves as the sample size increases with the rate of improvement decreasing

rapidly on sample sized above 20-30 cases per group. Figure 3 shows an example of the variation of the median across multiple samplings.

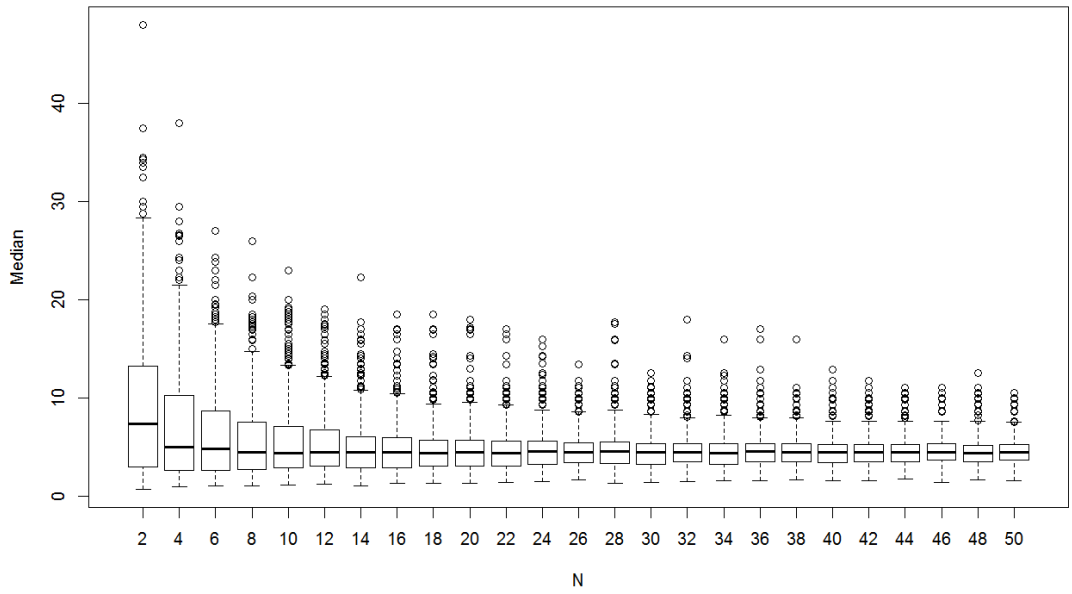



**Figure 3.** Boxplot of the distribution of median values when performing replicative sampling (n=1000) of sample sizes N=2, 4, 6...50 for the substance Citalopram (Group A cases). The reference median value in the complete data set is 4.5. The deviation from the expected median decreases as the sample size increases. In the boxplot, the whiskers are 1.5 times the interquartile range (IQR), and outliers are shown as circles. Adapted from paper IV.

Paper IV also present the number of detections needed in the in the forensic database in order to include a single detection in group A, group B or group C (accounting for detections "lost" in application of inclusion/exclusion criteria and manual review). Across all substances an average of 5.3 detections were needed in the database to include a single case in a group. As a general rule >300 detections would be needed to have a reasonable chance of obtaining 20 detections in group A, group B and group C in a best case scenario. However based on the distribution between the number of cases in different groups in paper I-IV, it can be assumed that group A cases are more rare than group C, requiring a larger pool of detections to obtain enough cases in each group.

# 5 DISCUSSION

## 5.1 WHAT IS CONSIDERED A FATAL INTOXICATION IN THE FORENSIC SETTING?

When using forensic toxicological results to diagnose fatal intoxication, and then using the diagnosed fatal intoxications as a basis for what is considered a fatal concentration there is a risk of circular reasoning. It would be a mistake to ignore that circular reasoning is a problem regarding to postmortem reference values. The question is; How can you diagnose fatal intoxication in the absence of forensic toxicological evidence?

The only way available is to supplement the toxicological interpretation with circumstantial information (e.g. police reports, pictures from the scene of the death and medical history) and the results from the forensic autopsy (including histological examination of relevant tissues), and evaluate the concentration in light of this supplemental information, as is advised in reviews of postmortem toxicology [10, 47, 55]. In the present papers, we also present control concentrations (group C), which suffers much less from the impact of circular reasoning than intoxication cases. Using the concentrations from group C together with the concentrations from the intoxication groups and circumstantial information allows for a more balanced and secure evaluation. Additionally, the diagnosis of fatal intoxication should be considered a diagnosis of exclusion employed only when conflicting causes of death have been ruled out.

However, the forensic toxicological results are of great importance when diagnosing a fatal intoxication, and thus a certain extent of circular reasoning cannot be avoided.

## 5.2 THE IMPORTANCE OF PREANALYTIC AND ANALYTIC VARIATION

When using a reference concentration to evaluate the concentration in an individual case it is important to consider the sources of variation.

A broad spectrum of preanalytic sources of variation is of importance in postmortem forensic toxicology [47, 55]. It is difficult to predict the extent to which each of them impact the final analytical result. However, with regard to postmortem redistribution Zilg *et al* [46] have shown that the ratio between central and peripheral blood for various substances has a large variation (0.2-4.6), which is also dependent on the postmortem interval. While the extent of postmortem redistribution varies between substances, between individual cases and with the postmortem interval [32, 46] its impact is profound.

Analytical factors are also of importance, but are often less pronounced than the preanalytical factors. Guidelines [102] and published reviews [103] recommend a maximum bias of ± 15-20% at each concentration and a precision of 15-20%.

In a sufficiently large group of cases both preanalytical and analytical variations balance each other out. However, with regard to an individual case it is important to take both sources of variation into account in evaluation. Circumstances of the individual case (e g age, hospital care and tolerance) need also be taken into account.

### 5.2.1 Limit of quantification (LOQ)

One analytic factor that warrants attention is the limit of quantification (LOQ). Depending on the LOQ the population of cases, especially in group C (controls), can accrue a bias.

A study by Jonasson and Saldeen [104], using data from the same database as paper I-IV, only included cases in which the femoral blood citalopram concentration was >0.7 µg/g (which was considered as indicative of an overdose). Of the 22 included cases in the study, citalopram was considered as not contributing to the death in 8 cases. The median concentration among those 8 cases was 1.08 µg/g (range 0.7-1.5 µg/g). Compared to the citalopram group C concentrations in paper IV (median 0.3 µg/g) the difference is substantial. However, paper IV included all cases allowed by the LOQ (≥ 0.05 µg/g). The high cut-off in the study by Jonasson and Saldeen shifted their non-intoxication cases concentration range upward.

Similar shifts, but on a smaller scale, can occur when the differences in LOQ are less pronounced. Depending on where the LOQ is in relation to the therapeutic window of a substance a number of "normal" concentrations can either be included or excluded, which can impact the total concentration distribution of the included cases.

## 5.3 THE IMPACT OF SAMPLE SIZE ON THE RELIABILITY OF POSTMORTEM REFERENCE VALUES

While the robust review in the present papers serves to bolster the reliability of the classification (i.e. intoxication or control) in our included cases, the issue of low number of detections cannot be ignored and must be taken into account in evaluation. Special care must be taken with small sample sizes <5, which includes a large amount of available case reports and many of the substances included in paper I-IV.

Of the 41 substances presented in paper I-III, only 12 have >5 cases in each group and only 9 have >10 cases in each group. Of the 13 substances in paper IV, 9 have >5 cases in each group and 6 have >10 cases in each group. As can be seen the amount of cases are often low with respect to power and type I error even in the large material used in the present studies.

As mentioned previously the sample size and power required in postmortem toxicology is rarely discussed in the literature. In general it has been stated the sample sizes are often low (<10) and that large populations are often needed in order to understand associations between causes and effects [75], which is confirmed by the findings of paper IV.

With regard to precision it is difficult to provide a general guideline. For some substances a larger uncertainty might be acceptable, especially if there is little overlap between fatal concentrations and controls. However, with small sample sizes it is hard to draw any meaningful conclusions with regard to a larger general population. Using citalopram as an example, a substance that has been featured in two publications using the same method as in paper IV [70, 71], we can see how the reference concentrations have evolved, as more data have been available (Table 4).

**Table 4.** Reference concentrations of citalopram (μg/g) across different publications using the same method.

| | **Druid *et al* [70] (1997)** | | | **Reis *et al* [71] (2007)** | | | **Paper IV (2019)** | |
|---|---|---|---|---|---|---|---|---|
| | n | $10^{th}/50^{th}/90^{th}$ percentile[a] | | n | $10^{th}/50^{th}/90^{th}$ percentile | | n | $10^{th}/50^{th}/90^{th}$ percentile |
| **Group A** | 8 | 3.4/7.0/10.5 | | 50 | 1.5/6.5/27 | | 70 | 1.09/4.45/21.23 |
| **Group B** | 13 | 0.7/1.1/4.7 | | 243 | 0.7/1.3/5.9 | | 377 | 0.7/1.4/9.04 |
| **Group C** | 71 | 0.1/0.6/1.1 | | 629 | 0.1/0.3/0.7 | | 916 | 0.1/0.3/0.7 |

a In groups were n = 4-9 in the paper by Druid et al [70] the lower quartile and upper quartile was used instead of the $10^{th}$ and $90^{th}$ percentile.

From a forensic perspective it is of special interest to know how high "normal concentrations" (group C) can be and how low lethal (group A) concentrations can go. Looking at Table 4 we can see that as the number of included cases increased the $10^{th}$ percentile of group A cases decreased (3.4 μg/g to 1.09 μg/g) and the $90^{th}$ percentile of group C also decreased (1.1 μg/g to 0.7 μg/g). Thus, if case circumstances were otherwise equal, a case with a citalopram whole blood concentration of 1.1 μg/g could have been judged as potentially normal in 1997 and as a single drug intoxication in 2019. Considering that such a large number of cases as we have retrieved for citalopram will not be available for most substances, it clear that the concentrations observed for substances with low number of A, B and C cases should be used with caution.

In summary the weight of evidence that is ascribed to a reference value must be based on the sample size (i.e to the extent to which the reference concentration can be said to reflect a larger population). In the case of small sample sizes there is a risk falsely attributing cases when based on postmortem reference concentrations alone, and rather a careful review of the background and circumstances surrounding death becomes particularly important.

## 5.4 WHAT CAN BE CONSIDERED A "GOOD" REFERENCE IN POSTMORTEM TOXICOLOGY?

What can be considered a good reference in postmortem toxicology depends on the type of information it provides.

As mentioned previously (see section 1.4.3) descriptive compilations, such as the one by Launiainen and Ojanperä [67], are proficient at answering whether a given concentration is high or low when compared to the total pool of detections. In order for this type of compilation to be useful it is important that the LOQ (limit of quantification) is low enough to cover a large segment of occurring concentrations. In addition the number of detections need to be high enough to provide a basis for generalization.

In evaluated compilations similar to those presented in this thesis there are three important main points to consider;

- Reliability of the classification. In a postmortem reference there should be a high degree of certainty that a case that is presented as an intoxication actually is an intoxication (and also that the reverse is true).
- Homogenity of sample collection. Since there are multiple pitfalls with regard to postmortem toxicology [55], precautions must be taken to minimize their impact. In order to minmize errors samples should all be from the same matrix (e.g whole blood) and from the same sample site (e.g the femoral vein). Peripheral blood is preferred in order to minimize the impact of postmortem redistribution [30, 34].
- Sample size. A novel finding in Paper IV was the impact of sample size on the reliability of the presented postmortem reference values. Thus in order to draw reliable conclusions from avaliable reference material a high sample size is prefered. Based on our data >10 cases in each group are strongly recommended and 20-30 are preferable. While there is no higher limit on the number of cases that can be included, the added benefit of additional cases decreases >30, and the importance of added precision in the reference concentrations must be weighted against the impact of other sources of error that are more difficult to control for (e.g postmortem interval and analytical variation) [10, 55].

If the above points are satisfactorily fulfilled reference compilations using similar methods to that which is presented in this thesis can be deemed reliable. As an example, the reference concentrations of thioridazine and ziprasidone (see Table 3) are both equally reliable with regard to the first two points, but the lack of sample size makes the resulting reference concentration of ziprasidone more unreliable.

Compared to descriptive compilations the method used in this thesis are better at providing answers to the range of concentrations that can be considered normal and fatal (i.e. how high concentrations can still be considered “normal” and how low concentrations can be considered fatal).

With the data on sample size provided in Paper IV caution must be taken when using case reports and small case series as a basis for evaluation, since a majority of these include <5 cases in total. While both the reliability of the case classification and sample collections might be adequate, the low number of cases introduces a large degree of uncertainty to the reference concentration. Thus the case can be made that the primary value of case reports might not be the concentrations presented, and focus should perhaps be on other aspects such as reporting novel interactions, side effects or unexpected circumstances [105]. Case reports can also function as a starting point for further research [106].

## 5.5 STRENGTHS

While the field of postmortem toxicology comprises a complicated problem with regard to evaluation, the approach to reference values used in the included papers features several strengths.

The size of the database from which the study population is derived is large and features information from both forensic pathology and forensic toxicology. The large size of the study populations allows for application of strict selection criteria. It is equally important that both the intoxication cases and control cases are correctly identified, and hence by starting with a large material, there is more opportunities for excluding cases whenever the circumstances are not clear cut to minimize sources of error. While other studies use a similar approach [39-42, 72-74], there are slight differences in the process of case selection, with the present papers using multiple reviewer process and consensus meetings before inclusion as well as a focus on the immediate cause of death and the exclusion of incapacitation in the postmortem control cases.

Another strength is that the autopsy sampling method is standardized and only femoral blood is included, reducing the problem with postmortem redistribution.

Lastly the process used and the wealth of available information in each case enables a high confidence in the classification of cases, both in cases of intoxication and controls. The access to the autopsy report in all cases allows us to e.g. check the extent of possible contaminating injuries and to rule out competing cause of death in unclear cases. Similarly, the access to the police reports and, in a proportion of the cases, medical records allows for scrutinizing the circumstances surrounding death.

## 5.6 LIMITATIONS

As mentioned above there is a risk that circular reasoning impacts the generation of reference values. Having said that, this problem is reduced with the application of the strict inclusion and exclusion criteria and manual review.

One disadvantage of the process is that it is time consuming. Using multiple rounds of evaluation including a multiple reviewer consensus requires both time and manpower. Another disadvantage of this approach is that it requires many detections of a substance to reach a significant number of certifiable non-intoxications deaths and intoxication cases.

In addition it is important to remember that all postmortem cases in the present papers are extracted from a forensic pathological population. Thus there are factors of age, sex and burden of disease that may not be applicable to the general population. This might, in turn, impact the susceptibility to the adverse effects of ingested substances that could affect the resulting postmortem reference concentrations.

Lastly it is important to remember that there is a multitude of factors that may influence the blood drug concentration in each particular case [10, 47, 55]. Thus it is important to evaluate

postmortem concentrations not only with regard to reference concentrations, but also with regard to the unique circumstances in each case.

# 6 CONCLUSIONS

The method used to provide postmortem reference in postmortem femoral blood was presented along with suggested reference values for a total of 48 substances, providing support to the forensic pathologist in evaluation of postmortem toxicological results.

Novel information with regard to the relationship between sample size and precision, power and type I error was presented based on our data. These results underscore the importance of compilation and evaluation of sufficiently large populations in order to generate reliable reference concentrations.

# 7 FUTURE DIRECTIONS

As has been demonstrated by the calculations done in paper IV there is a need for updating reference concentrations for drugs where previous evaluations were based on small sample sizes. A target number would be a minimum of 20-30 cases in each group. However, as can be seen in paper IV, the number of detections needed to reach the desired number included is high. Higher degree of international cooperation in pooling toxicological data for research purposes could greatly aid the quality of postmortem reference concentrations.

Our current reference values do not take the chiral structure [107] of substances into account. For example, the concentrations of citalopram in paper IV are the total citalopram concentration, which is in turn is a racemate of R-citalopram and escitalopram. Escitalopram has been shown to be responsible for the serotonin reuptake and antidepressant effect of citalopram. In addition it has been shown that escitalopram alone has better effect than the same amount of escitalopram given in combination with R-citalopram. In effect R-citalopram inhibits the effect of escitaopram [108]. R-citalopram and escitalopram also seem to have different impact on the QTc interval [109]. Thus it may be that the concentration that can be considered normal and toxic might be different for escitalopram and total citalopram, which is not reflected in our current reference values. While the department of forensic toxicology does not yet use a chiral method for citalopram, introduction of such a method might generate data that may improve the interpretation of citalopram.

Paper I-IV only takes into account the parent compound of the studied substances. A previous study by Reis *et al* [71], using the same method for obtaining postmortem reference values as in the present papers, presented ratios of metabolite to parent compound in intoxications and controls. However, it is well known that active metabolites can and do play a role in the pharmacologic response and toxicity [110, 111]. One way to approach this could be to add concentrations of parent substance and metabolite together, as has been done with nitrazepam in paper II and IV (although done there for reasons of postmortem metabolism). However, when using the current reference values, active metabolites must be taken into account on an individual case-by-case basis.

The results from Paper III could not provide evidence for the link between the mode of lithium intoxication and the resulting intoxication concentrations. However, the underlying question of changes at a substances site of action that impact at which concentrations side effects occur is interesting. Because of tolerance [55] the concentration measured in the blood might not be the best measure of the effects of a drug. As an example, the respiratory depression caused by opioids is the result of action at respiratory control centers in the central nervous system [112]. Future research could combine results from hair analysis [60, 113, 114] and drug concentrations at its site of action to provide more accurate information when evaluating the effect of a particular intake of a drug.

It has been stated that 15-30%, and in some cases up to 95%, of drug metabolism and drug response are attributed to genetic factors [115]. From a forensic viewpoint differences in metabolic ability (i.e. fast vs. slow metabolizers) are of importance when determining the manner of death and difference in drug targets and drug transport can be of importance for determining fatal intoxication as a cause of death [116]. Differences in pharmacodynamic response to a given drug based on genetic factors will of course impact which concentrations that can be considered toxic and normal in different genetic populations. Thus, a future approach could be to try to differentiate cases of intoxication according to genetic polymorphisms.

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